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To study the effects of the common contaminants of hemoglobin solutions, red cell stroma, bacterial endotoxin, and denatured hemoglobin on the causes of the thrombotic lesions which have been reported in animal experiments after hemoglobin administration. Human blood mononuclear cells were isolated on Ficoll-Hypaque gradients and incubated with hemoglobin from the LAIR production facility, red cell stroma, bacterial endotoxin (E. Coli, Wittaker Bioproducts), and hemoglobin denatured by boiling. Incubations were performed separately and in combinations. Mononuclear cells were then lysed and assayed for procoagulant activity in a recalcification time assay. Only bacterial endotoxin and hemoglobin denatured by boiling increased the procoagulant activity of human blood mononuclear cells. Denatured hemoglobin mixed one part in eight with undenatured hemoglobin increased mononuclear cell procoagulant activity by more than ten-fold that of the undenatured hemoglobin control. The study suggests that denatured but not undenatured hemoglobin causes increased blood procoagulant activity which is though to be a marker of macrophage activation. These findings suggest a possible mechanism of toxicity of cell-free hemoglobins and the need for sensitive measures of hemoglobin denaturation.

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DENATURED HEMOGLOBIN INCREASES HUMAN BLOOD MONONUCLEAR CELL PROCOAGULANT EFFECT

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ABSTRACT

Purpose: To study the effects of the common contaminants of hemoglobin solutions, red cell stroma, bacterial endotoxin, and denatured hemoglobin on the causes of the thrombotic lesions which have been reported in animal experiments after hemoglobin administration.

Protocol: Human blood mononuclear cells were isolated on Ficoll-Hypaque gradients and incubated with hemoglobin from the LAIR production facility, red cell stroma, bacterial endotoxin (E. coli, Wittaker Bioproducts), and hemoglobin denatured by boiling. Incubations were performed separately and in combinations. Mononuclear cells were then lysed and assayed for procoagulant activity in a recalcification time assay.

Results: Only bacterial endotoxin and hemoglobin denatured by boiling increased the procoagulant activity of human blood mononuclear cells. Denatured hemoglobin mixed one part in eight with undenatured hemoglobin increased mononuclear cell procoagulant activity by more than ten-fold that of the undenatured hemoglobin control.

Conclusions: The study suggests that denatured but not undenatured hemoglobin causes increased blood procoagulant activity which is thought to be a marker of macrophage activation. These findings suggest a possible mechanism of toxicity of cell-free hemoglobins and the need for sensitive

measures of hemoglobin denaturation.

INTRODUCTION

The toxicity of cell-free hemoglobin (Hb) has been difficult to define because mechanisms of toxic action are unexplained and because trace contaminants are present in all Hb preparations. Understanding the interaction of Hb with the immune system has been a particular problem [1]. Fever and inflammatory lesions have been seen variably after administration of a variety of Hb products and in a number of testing situations [2]. The inconsistency of observed inflammatory toxicity has led to the frequent assumption that unrecognized contamination of the Hb products was responsible for the toxicity.

The common contaminants of cell-free Hb are red blood cell stroma, breakdown products of contaminating bacteria, and breakdown products of Hb itself. Cell wall phospholipids, bacterial endotoxin, and heme cause known toxicity.

We have attempted to determine if cell-free Hb separate from its contaminants increases human mononuclear cell procoagulant activity. We have tested for this activity after exposing cells to sterile, HPLC purified human Hb and combinations of Hb and freshly isolated stroma, isolated bacterial endotoxin, and denatured Hb. Denatured Hb and endotoxin cause the response, but native Hb or stroma do not.

MATERIALS AND METHODS

HbA_0 was isolated from stroma-free hemolysate (SFH) by high performance liquid chromatography (HPLC). The SFH was made by lysing outdated banked RBCs with hypotonic phosphate buffer and successive cross-flow filtering at 0.65 μm and .011 μm (300KD cut-off). HPLC was performed on a 10 x 90 cm Mono-Q column with saline gradients on a Waters Kiloprep at 4°C. The HbA_0 was formulated as a 6 g/dl solution in Ringer's acetate. Endotoxin was less than 0.06 EU/ml and organic phosphate was less than 1 μg/ml.

Human mononuclear cells were isolated from heparinized venous blood first as buffy coat and then as enriched buffy coat by centrifugation and finally as mononuclear cell suspension on Ficoll-histopaque density gradients. Cells were washed and maintained in HEPES-buffered saline. Cell isolation was confirmed by differential counting, α-naphthyl acetate esterase staining, and trypan blue staining.

RBC stroma were prepared from the precipitate of trichloromethane extracted, triply frozen, fresh whole blood. The precipitate was washed and suspended in HEPES saline.

Bacterial endotoxin from *E. coli* 0127:B8 was purchased from Sigma Chemical Co., St. Louis, MO.

Denatured hemoglobin was made by removing 1/8 part (0.5 ml of 4 ml) of the HbA_0 solution, heating it to 100°C for 10 minutes, and adding it back to the undenatured fraction.

Isolated cell fractions were divided and mixed with buffer or contaminant, and then divided again, with half of the material frozen immediately and the other half incubated in parallel with its control for 20 hours at 37°C. This pattern of parallel incubation with preincubation controls was repeated for cells from each donor (n=3) with HbA_0 alone and with HbA_0 and added endotoxin, stroma, and denatured Hb.

Measurement of procoagulant activity was performed with freeze-thawed, n-octyl-α-D-glucopyranoside solubilized, and sonicated cell suspensions in a one-stage clotting time measured in a fibrometer (Dataclot 2, Helena Labs, Beaumont, TX). Activity was measured as units of thromboplastic activity against a rabbit brain standard. Procoagulant activity ratios were calculated from clotting time ratios normalized for protein content as (Post-incubation activity with Hb & contaminant/Post-incubation activity with vehicle alone)/(Pre-incubation activity with Hb & contaminant/Pre-incubation activity with vehicle alone).

RESULTS

Preliminary experiments showed a marked variability in mononuclear cell procoagulant activity among freshly isolated cells of different donors. The increase in activity with exposure to Hb or a contaminant was thus expressed as an activity ratio with the unincubated sample as the control. The increase in procoagulant activity with increasing endotoxin exposure was also dose dependent, but varied among donor's cells.

In this system clotting time did not increase with incubation for 20 hr with HbA_0. Data from a single patient are shown in Figure 1.

Procoagulant activity increased with incubation with endotoxin and denatured Hb. Data from a single patient are shown in Figure 2.

DISCUSSION

Mononuclear cell procoagulant activity is believed to be tissue factor protein which is normally present in small amounts inside cells and is synthesized and externalized in response to activating stimuli. The experimental design was developed to allow control of the variability in mononuclear cell procoagulant activity expression between white cell donors and to differentiate increases in activity associated with handling from those associated with exposure to potentially stimulating agents.

We found that incubation with human Hb that had been purified by HPLC and that contained concentrations of bacterial endotoxin below 0.06 EU/ml did not increase the human mononuclear cell procoagulant effect. This result is at variance with previous work from our lab [3].

Bacterial endotoxin and denatured Hb strongly and independently increased the human mononuclear cell procoagulant effect. Endotoxin is known to activate this response. Denatured hemoglobin separate from hemoglobin has not previously been reported to cause this reaction. Whether a specific reaction to the denatured Hb or a nonspecific reaction to denatured protein is responsible is unknown.

RBC stroma did not independently increase the procoagulant effect. Stroma has been reported as toxic in the past [4].

We conclude that Hb denaturation and therefore storage stability may be critical issues in Hb safety. While good manufacturing practice can insure low levels of contamination with phospholipids and bacterial endotoxin, proper post-manufacture handling is necessary to prevent the development of hemoglobin breakdown products. More sensitive measures of Hb denaturation should be developed.

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The opinions and assertions contained herein are the private views of the authors and are not to be construed as official nor do they reflect the views of the Department of the Army or the Department of Defense (AR 360-5).

Human Subjects participated in these studies after giving their free and informed voluntary consent. Investigators adhered to AR 70-25 and USAMRDC Reg 50-25 on the use of volunteers in research.

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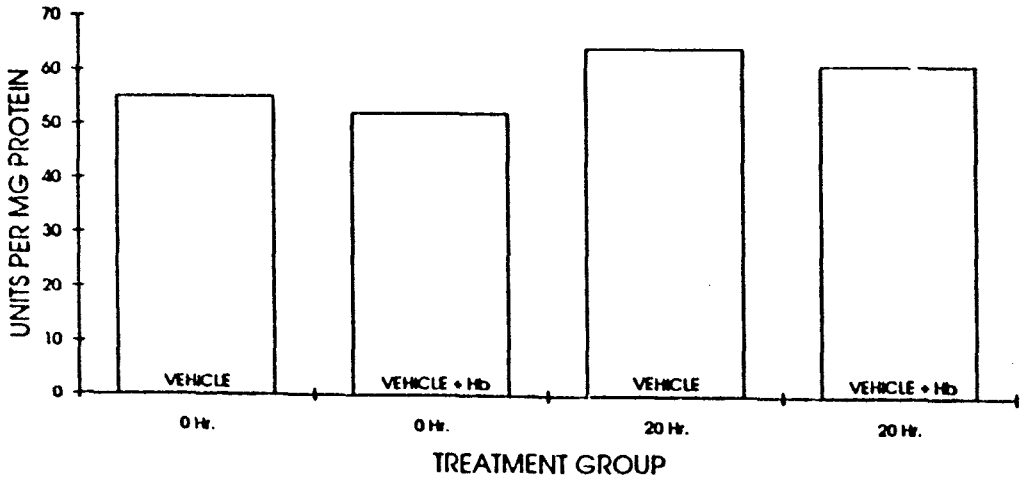
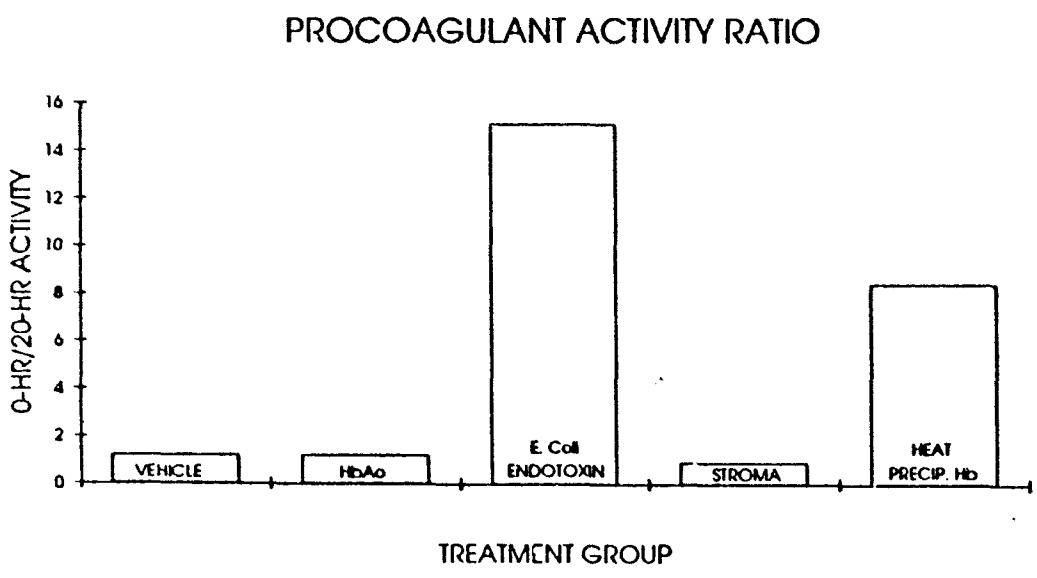
Figure 1. Procoagulant activity of human monocytes from one donor exposed to HbA_0 and saline vehicle for 0 and 20 hours. The minimal increase with incubation appears to be nonspecific because a similar increase occured with exposure to the saline control. The activity ratio calculated from these measures is about 1.2.

Figure 2. Procoagulant activity ratio for the cells of one donor after the cells were exposed for twenty hours to vehicle, HbA_0, and Hb and contaminants as listed. Endotoxin and heat denatured Hb consistently caused elevations about 10 times baseline values.

PROCOAGULANT ACTIVITY OF MONOCYTES




PROCOAGULANT ACTIVITY RATIO
0-HR/20-HR ACTIVITY
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VEHICLE
HbAo
E. Coli ENDOTOXIN
STROMA
HEAT PRECIP. Hb
TREATMENT GROUP